Developmental Cell, Vol. 2, 29–40, January, 2002, Copyright ©2002 by Cell Press

Rca1 Inhibits APC-Cdh1^{Fzr} and Is Required to Prevent Cyclin Degradation in G2

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Summary

We demonstrate that Rca1 is an essential inhibitor of the anaphase-promoting complex/cyclosome (APC) in *Drosophila*. APC activity is restricted to mitotic stages and G1 by its activators Cdc20-Fizzy (Cdc20^{Fzy}) and Cdh1-Fizzy-related (Cdh1^{Fzr}), respectively. In *rca1* mutants, cyclins are degraded prematurely in G2 by APC-Cdh1^{Fzr}-dependent proteolysis, and cells fail to execute mitosis. Overexpression of Cdh1^{Fzr} mimics the *rca1* phenotype, and coexpression of Rca1 blocks this Cdh1^{Fzr} function. We show that Rca1 and Cdh1^{Fzr} are in a complex that also includes the APC component Cdc27. Previous studies have shown that phosphorylation of Cdh1 prevents its interaction with the APC. Our data reveal a different mode of APC regulation by Rca1 at the G2 stage, when low Cdk activity is unable to inhibit Cdh1^{Fzr} interaction.

Introduction

The anaphase-promoting complex/cyclosome (APC) is a multisubunit ubiquitin ligase that targets several mitotic regulators for degradation and thereby triggers an exit from mitosis (see Morgan, 1999; Zachariae and Nasmyth, 1999 for review). The APC and many of its target proteins are present throughout the cell cycle. However, its activity is restricted to mitotic stages and G1. This is achieved by the cell cycle-dependent association of two WD40-repeat-containing proteins, called Cdc20 and Cdh1 in *S. cerevisiae*, respectively (Inbal et al., 1999; Visintin et al., 1997; Yamaguchi et al., 1997). These proteins associate only transiently with the APC but are required for full APC activity. Cdc20 and Cdh1 homologs have been identified in a variety of different organisms and come with different names. In *Drosophila*, these are encoded by the *fizzy* (*fzy*) and *fizzy-related* (*fzr*) genes, respectively, and we will refer to the fly proteins as Cdc20^{Fzy} and Cdh1^{Fzr} (Dawson et al., 1995; Sigrist and Lehner, 1997).

Binding of Cdc20 and Cdh1 to the APC is differentially regulated. APC-Cdc20 activity is present during mitosis and initiates the metaphase-anaphase transition. The association of Cdc20 with the APC requires phosphorylation of at least one subunit of the APC (Kramer et al., 2000; Shteinberg et al., 1999). Several mitotic kinases have been implicated in this phosphorylation. The dependency of APC phosphorylation on Cdc20 binding ensures that APC-Cdc20 is only active during mitosis. During prophase and prometaphase, APC-Cdc20 activity is furthermore restrained by the spindle checkpoint. This system monitors the presence of unattached kinetochores. Until kinetochores are bound by spindles, they serve as an assembly point for active Mad2 protein. Mad2 binds to Cdc20 and inhibits APC activity (see Shah and Cleveland, 2000 for review). Once all kinetochores are attached and chromosomes are aligned on the metaphase plate, Mad2 inhibition of APC-Cdc20 activity is released.

Cdh1 is found in association with the APC during later stages of mitosis and G1. This interaction depends on the phosphorylation status of Cdh1 (Kramer et al., 2000; Zachariae et al., 1998). Only unphosphorylated Cdh1 is able to bind to and activate the APC (Kotani et al., 1999; Kramer et al., 2000). Cdk1 and Cdk2 mediate Cdh1 phosphorylation. Thus, only during stages of low Cdk kinase activity will Cdh1 activate the APC (Blanco et al., 2000; Jaspersen et al., 1999; Listovsky et al., 2000; Sorensen et al., 2001). These requirements are fulfilled during later stages of mitosis, when APC-Cdc20 has induced the degradation of mitotic cyclins, and during G1, when Cdk kinase activity is low. However, the G2 stage is also characterized by low Cdk kinase activity. How Cdh1-dependent APC activity is prevented in these situations has not been addressed so far.

The mitotic cyclins in *Drosophila* (Cyclin A [CycA], Cyclin B [CycB], and Cyclin B3) are stable in interphase, degraded during mitosis, and continue to be unstable throughout G1. Cdc20^{Fzy} is required for mitotic cyclin destruction at the metaphase-anaphase transition and is thought to mediate the bulk of cyclin degradation in the first 16 cell cycles in *Drosophila* (Dawson et al., 1993; Sigrist et al., 1995). Mutants in *fzy* arrest in metaphase of cell cycle 16 when the maternal supply of Cdc20^{Fzy} is exhausted (Dawson et al., 1993; Sigrist et al., 1995). Overexpression of *fzy* does not cause abnormal cyclin destruction. Thus, Cdc20^{Fzy} is not able to activate the APC at other cell cycle stages (Sigrist and Lehner, 1997). This likely reflects the inability of Cdc20 to interact with unphosphorylated APC (Kramer et al., 2000; Shteinberg et al., 1999).

Mitotic cyclins remain unstable during G1, mediated by APC-Cdh1^{Fzr}-dependent degradation (Sigrist and Lehner, 1997). The first G1 phase during embryogenesis is not established in *fzr* mutants, and cells perform an additional S phase, presumably triggered by the S phase activity of the CycA/Cdk1 complex (Sigrist and Lehner, 1997; Sprenger et al., 1997). *Cdh1^{Fzr}* mRNA expression cannot be detected during the cellular blastoderm stages, but low levels of Cdh1^{Fzr} are presumably present (J. Raff, personal communication). High levels of Cdh1^{Fzr} are expressed during stage 11 of embryogenesis, when most cells are in the 16th cell cycle, shortly before they enter the first G1 phase. In contrast to Cdc20^{Fzy}, APC activation by Cdh1^{Fzr} can be induced ectopically by its overexpression (Sigrist and Lehner, 1997). During embryogenesis, this results in degradation of mitotic cyclins in G2 of cell cycle 16 and a failure to execute mitosis 16.

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Here, we demonstrate that Rca1 is an important negative regulator of Cdh1^{Fzr}-dependent APC activity. The *rca1* gene was initially identified as a dominant modifier of the *rux* eye phenotype (Dong et al., 1997). Rux is a cell cycle regulator important for the downregulation of mitotic cyclin/Cdk1 activity during mitosis and G1 (Foley et al., 1999). Homozygous *rca1* mutants are embryonic lethal and arrest in G2 of cell cycle 16 with a phenotype very similar to *CycA* loss-of-function mutants, hence its name, r*egulator of cyclin* A (*rca1*). Overexpression of *rca1* during eye development induces ectopic S phases and results in elevated CycA levels. However, in *rca1* mutants, Dong et al. found no difference in CycA protein levels during embryonic cycles 14–16.

A potential homolog of *rca1* was recently identified in vertebrates. This protein, *e*arly *m*itotic *i*nhibitor (Emi1), interacts with Cdc20 and prevents its association with the anaphase-promoting complex (Reimann et al., 2001). Both proteins contain a nuclear localization signal, a putative F-box, and a region with a characteristic cystein spacing, that could serve as a potential zinc binding domain (van der Reijden et al., 1999).

Here, we show that Rca1 specifically inhibits Cdh1^{Fzr}-dependent APC activity. In the absence of *rca1* function, mitotic cyclins are prematurely degraded, and cells fail to enter mitosis. This phenotype is reminiscent of the phenotype after overexpression of Cdh1^{Fzr}. Double-mutant analysis demonstrates that premature cyclin destruction in *rca1* mutants is mediated by Cdh1^{Fzr}. Furthermore, we show that Rca1 can block the effects of Cdh1^{Fzr} overexpression, supporting the notion that Rca1 inhibits Cdh1^{Fzr}-dependent APC activity. Coimmunoprecipitation experiments reveal that Rca1 and Cdh1^{Fzr} are in a complex that also contains the APC component Cdc27. Collectively, our data show that Rca1 is a negative regulator of Cdh1^{Fzr}-dependent APC activity. We suggest that a similar function is required in all cells in which kinase activity is low during G2 to prevent a premature activation of the APC by Cdh1.

Results

Mitotic Cyclins Disappear Prematurely in *rca1* Mutant Embryos

Embryos mutant for *rca1* undergo normal embryonic development and cell proliferation until the extended germband stage (Dong et al., 1997). At this time, epidermal cells in wild-type (wt) embryos undergo the 16th mitotic cycle and then enter a G1 phase for the first time of embryogenesis. *rca1* mutants fail to perform this division, resulting in embryos with reduced cell number compared with those of wt (Dong et al., see also Figures 1 and 5). No mitotic spindles are seen at the time when cells would normally undergo mitosis 16 (Dong et al., 1997) and the DNA is decondensed (Figure 1E). Thus, *rca1* mutants arrest during interphase of cycle 16. These features are identical to those of *CycA* mutants (Lehner and O'Farrell, 1989), but it was reported that no differences in CycA levels were detectable in *rca1* mutant embryos (Dong et al., 1997). In contrast, we find CycA markedly reduced shortly before embryos would normally enter mitosis 16 (Figure 1D). In wt, this mitosis occurs in a stereotypic way that is reflected in the degradation pattern of CycA. This pattern is segmental (Figure 1A), and cells expressing high levels of CycA (G2 cells of cycle 16) can be distinguished from G1 cells with low levels of CycA (Figure 1C). In *rca1* embryos, only uniformly low levels of CycA can be detected in the epidermis at this stage (Figure 1D), and mitotic figures were seen only occasionally (Figures 1E and 1F, arrowheads). Most of the cells show a size typical for cell cycle 16 with the DNA in a decondensed state (Figure 1E).

To determine when *rca1* mutant cells deviate from the wt situation, we examined embryos that lack functional Rca1 in every other segment. We constructed a haemagglutinin (HA)-tagged *rca1* cDNA under the control of the UAS promoter. HA-Rca1 was expressed in every second segment in the *rca1* mutant background by a *paired*-Gal4 (*prd*-Gal4) driver line (Brand and Perrimon, 1993). HA-Rca1 was detected using HA antibodies (Figure 1G). High magnifications showed that Rca1 is nuclear (Figure 1H), confirming a nuclear localization sequence between amino acids 115 and 133. HA-Rca1-expressing segments underwent mitosis 16, visualized by staining for phosphorylated histone 3 (PH3; Figures 1I and 1J). This demonstrates that HA-Rca1 was able to rescue the mitotic failure in *rca1* mutants. Indeed, *HA-rca1*-expressing segments had almost twice as many cells as the mutant segments, when analyzed in older embryos (Figures 1K and 1L).

We then analyzed the degradation pattern of two mitotic cyclins, CycA and CycB (Figures 1M–1P). In HA-Rca1-expressing segments, CycA (Figures 1M and 1N) and CycB (Figures 1O and 1P) levels were high compared with *rca1* mutant segments. No differences were detected in slightly younger embryos between adjacent segments, and both cyclins accumulated normally at the beginning of interphase 16 (see Supplemental Data, Figure S1 [www.developmentalcell.com/cgi/content/full/2/1/29/DC1]). We therefore conclude that the disappearance of mitotic cyclins in *rca1* mutant cells is caused by their premature destruction in late interphase of cell cycle 16. The remaining cyclin levels are apparently not sufficient to drive cells into mitosis.

CycA is essential for mitosis, but *rca1* mutants might also lack other components required for mitotic induction. To address this question, we expressed additional CycA in *rca1* mutants using an UAS-HA-CycA construct and the *prd*-Gal4 driver line (Figures 1Q and 1S). This construct does not disturb mitotic progression in a wt background and is able to rescue the *CycA* mutant phenotype (data not shown). In the *rca1* mutant background, HA-CycA-expressing segments had higher cell numbers (Figure 1S). Thus, overexpression of CycA can overcome the mitotic block of *rca1* mutant embryos. This shows that CycA is the only essential mitotic function missing in the *rca1* mutants.

rca1 Mutant Cells Fail to Proliferate

The defect in *rca1* mutants can be observed in cell cycle 16 at a time shortly before the first G1 phase in *Drosophila* embryogenesis. To test if *rca1* function is restricted to embryogenesis, we generated *rca1* mutant cells in imaginal disc epithelia using the FLP/FRT system (Xu and Rubin, 1993). Cells homozygous for *rca1* were identified by the absence of a nuclear GFP that was expressed from the sister chromosome.

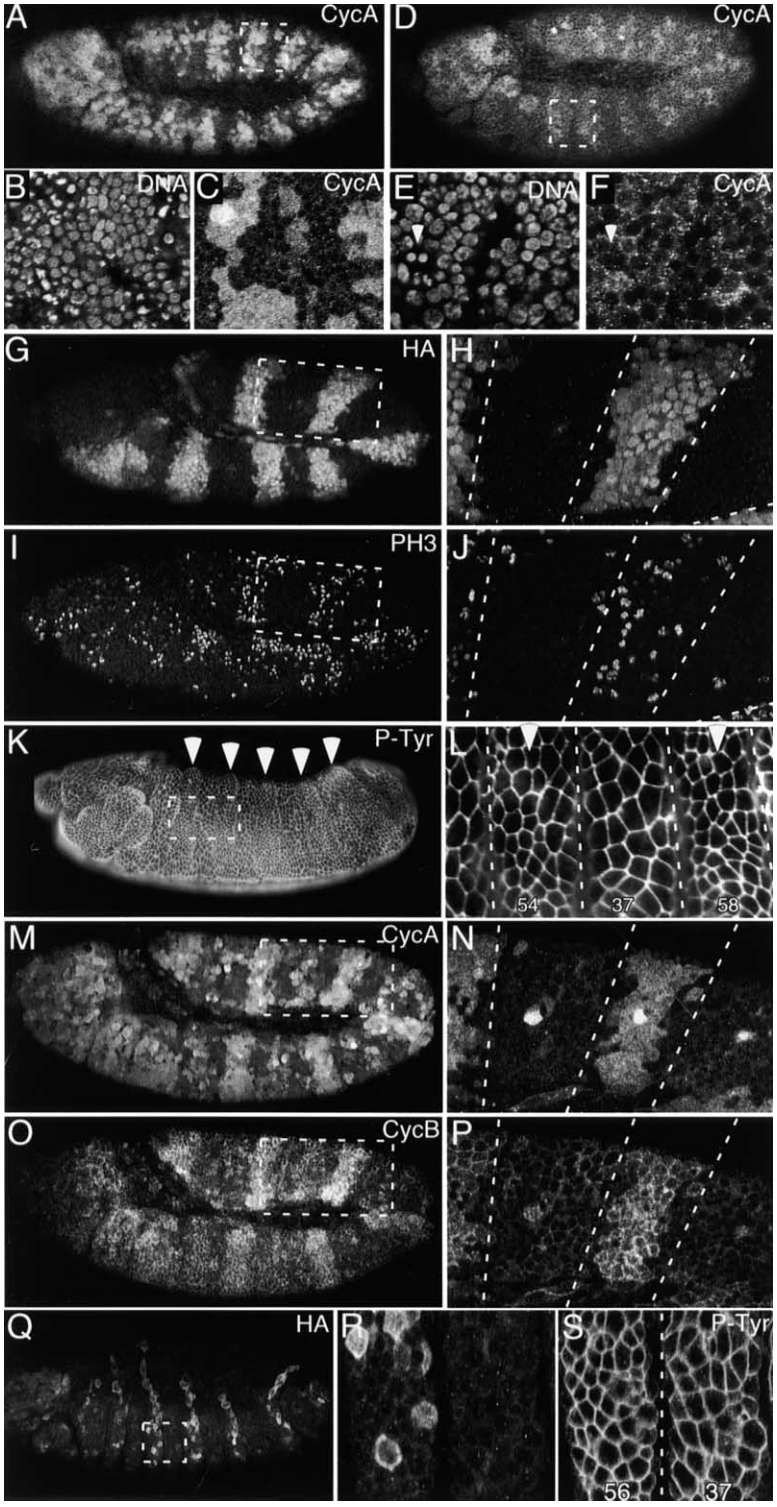

Figure 1. Mitotic Cyclins Disappear Prematurely in *rca1* Mutant Embryos

(A–F) Cyclin A disappears prematurely in *rca1* mutant embryos. Wt (A–C) and *rca1* mutant embryos (D–F) stained for CycA (A, C, D, and F) and DNA (B and E). At stage 11, epidermal cells show a segmental CycA pattern (A), and cells with high CycA levels (G2 cells of cycle 16) can be distinguished from cells with low levels (G1 cells of cycle 17; [B and C]). In addition, mitotic cells can be seen containing condensed DNA (B). In *rca1* mutants, CycA is visible (D and F) but present only at uniformly low levels in the epidermis (F). Most cells have a cell size typical for cycle 16 (compare [C] and [F]), and mitotic figures are only observed occasionally (arrowheads in [E] and [F] mark a telophase).

(G–L) HA-Rca1 can rescue the *rca1* mutant phenotype. *rca1* mutants expressing HA-Rca1 in alternating segments induced by a *prd*-Gal4 driver line. At stage 11, HA-Rca1 is strongly expressed (HA antibody staining in [G] and [H]) and mitotic cells, labeled with an antibody against phosphorylated histone 3 (PH3), are mainly restricted to the HA-Rca1-expressing segments (I and J), indicating that HA-Rca1 can restore mitosis 16 in *rca1* mutant cells. (K and L) A stage 13 embryo was stained with an antibody against phosphotyrosin (P-tyr) to visualize the outline of the cells. HA-Rca1-expressing segments (white arrowheads in [K]) show higher cell densities than neighboring segments (L), demonstrating that HA-Rca1 can rescue the cell division phenotype of *rca1* mutant cells.

(M–P) CycA and CycB are degraded prematurely in cells lacking Rca1 function. In a stage 11 embryo, CycA (M and N) and CycB (O and P) levels are lower in *rca1* mutant segments that do not express HA-Rca1.

(Q–S) HA-CycA can rescue the *rca1* mutant phenotype. HA-CycA expression in every second segment in a stage 13 *rca1* mutant embryo visualized by HA antibody staining (Q and R). P-tyr staining of the same embryo (S) shows that segments overexpressing HA-CycA have a higher cell density than nonexpressing segments.

Dashed lines indicate the approximate borders of *prd*-Gal4-expressing and -nonexpressing segments. Anterior is to the left and dorsal is up in all embryos and magnifications shown. Boxes indicate the magnified region. The numbers of cells in an outlined area is indicated.

In these imaginal discs, we could only see small *rca1* mutant clones, while their twin clones were significantly larger (Figure 2). Thus, *rca1* mutant clones have a growth disadvantage. Presumably, mutant cells have a limited proliferation potential and stop dividing after a few cell divisions. The clone size of mutant cells is also affected by cell elimination, since we find clones that contain two copies of the GFP gene without the corresponding *rca1* mutant twin clone (Figure 2). In all imaginal discs analyzed ($n = 30$), clones were found throughout the disc and were not restricted to a particular region. The clones shown in Figures 2C and 2E are partly within the ZNC (zone of nonproliferating cells) but also extend to the area in which cells normally proliferate. In the ZNC,

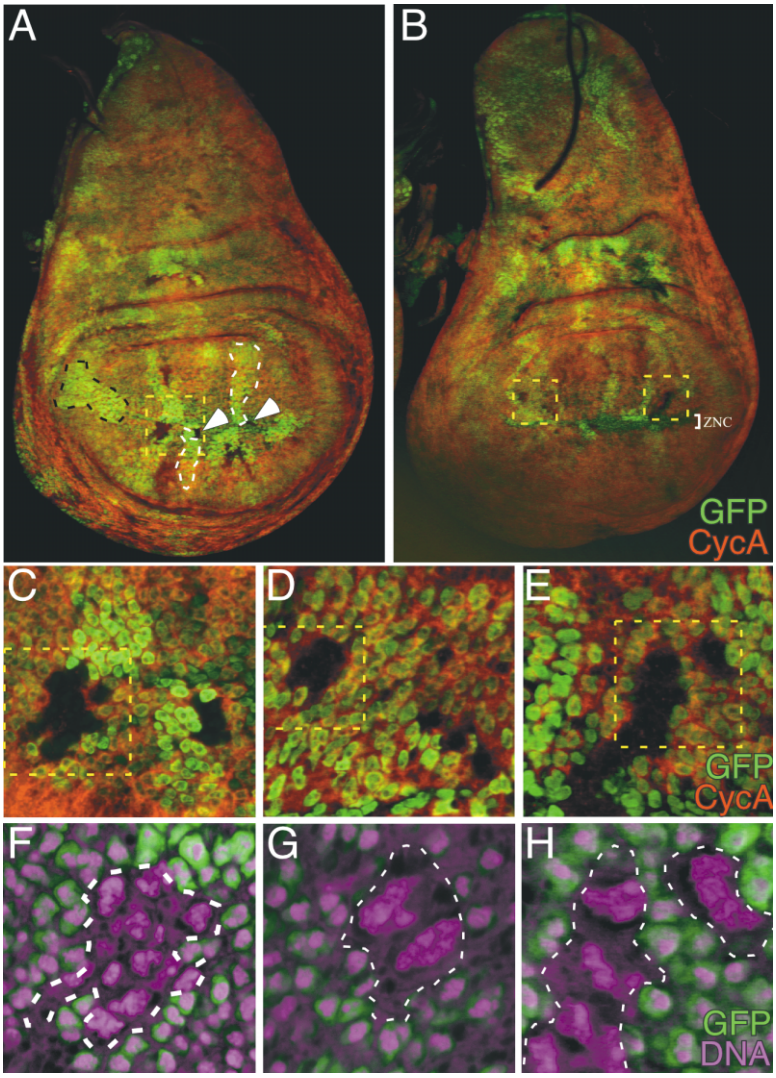

Figure 2. *rca1* Mutant Cells Fail to Proliferate

(A–H) Third instar wing imaginal discs containing *rca1* mutant clones induced by FLP/FRT-mediated mitotic recombination. The FLP recombinase was under the control of a heatshock promoter. Clones were induced at 48 hr after egg deposition (AED) and analyzed at 120 hr AED. $rca1^{-/-}$ clones are marked by the absence of GFP, whereas the $rca1^{+/+}$ twin clones show a bright GFP fluorescence, since they contain two copies of the GFP transgene. Clones can be found throughout the disc and are not restricted to particular regions. GFP-negative clones (two are indicated with arrowheads in [A]) are smaller than their twin clones (outlined with white dashed lines in [A]), showing that *rca1* mutant cells had a growth disadvantage. Some clones containing two copies of the GFP transgene lack the corresponding *rca1* mutant clone (an example is outlined by a black dashed line in [A]). This indicates that $rca1^{-/-}$ clones are eventually eliminated. CycA staining (red channel) reveals that the GFP-negative cells ($rca1^{-/-}$) have lower CycA levels than the surrounding GFP-positive ($rca1^{+/-}$ or $rca1^{+/+}$) cells. Nuclei of $rca1^{-/-}$ cells are enlarged and appear to contain higher amounts of DNA (magenta channel) compared with the surrounding cells (F–H). The zone of nonproliferating cells, a region in which cells are arrested, is marked by a bracket. Magnified regions are indicated by yellow dashed boxes.

anterior cells in the central region as well as posterior cells arrest in G1 and display low CycA levels (Johnston and Edgar, 1998). The clone in Figure 2D is completely outside the ZNC. Staining for CycA revealed that all mutant clones had reduced CycA levels (Figures 2A–2E). Thus, *rca1* function is generally required for maintaining mitotic cyclin levels in proliferating cells. Interestingly, nuclei of the mutant cells were enlarged and appear to contain higher DNA levels (Figures 2F–2H). However, quantification on single cells would be required to determine the exact DNA content of these cells.

In conclusion, clonal analysis shows that *rca1* function is not only needed during embryogenesis, but that it is a general factor required for proliferation.

Overexpression of Rca1 Does Not Disturb Cell Cycle Progression

To see whether Rca1 overexpression influences normal cell cycle progression, we first used the *prd*-Gal4 driver line to express HA-Rca1 in alternating segments. We used an Rca1 antibody to detect the expressed transgene. The antibody recognized Rca1 in Western blots from embryonic extracts (data not shown) and overexpressed HA-Rca1 in embryos (Figure 3), but it fails to detect the endogenous protein, probably due to relatively low expression levels.

We compared CycA levels and cell cycle progression in segments that overexpress HA-Rca1 with wt segments. There was no deviation in CycA protein levels or cell cycle progression between neighboring segments (Figures 3A–3E). Thus, Rca1 does not promote CycA stability when overexpressed. In addition, the pattern of CycA degradation during mitosis was not changed by elevated Rca1 levels. CycA degradation starts in metaphase and persists through anaphase and telophase (Lehner and O'Farrell, 1989). Metaphase cells with CycA as well as those in which CycA is already degraded are visible in the HA-Rca1-overexpressing segment (Figures 3D and 3E, arrowheads). In telophase cells, CycA is completely degraded (Figure 3C–3E, brackets).

We next used the *arm*-Gal4 driver line that forces ubiquitous expression in all embryonic and imaginal tissues (Sanson et al., 1996). High levels of HA-Rca1 are present in embryos undergoing the 15th cell cycle (Figure

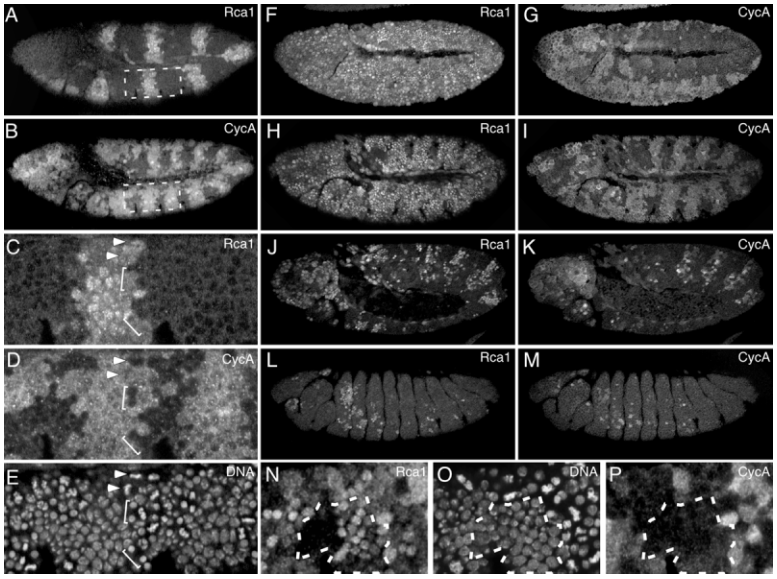

Figure 3. Overexpression of Rca1 Does Not Disturb Cell Cycle Progression

(A–E) HA-Rca1 overexpression using the *prd*-Gal4 driver line. (A and C) Stage 11 wt embryo stained with the Rca1 antibody. CycA (B and D) and DNA staining (E) of the same embryo reveals no difference in CycA levels or in the timing of CycA degradation between HA-Rca1-expressing and nonexpressing segments. The arrowheads in (C), (D), and (E) mark metaphase cells. The more dorsal cell has already degraded CycA, whereas it is still present in the ventral one. Brackets mark telophase cells in which CycA is absent. HA-Rca1 protein is present throughout the cell cycle, and degradation at any specific stage cannot be observed.

(F–P) HA-Rca1 overexpression using the *arm*-Gal4 driver line. (F) High levels of HA-Rca1 are present already during the 15th cell cycle, but no changes in the normal mitotic degradation pattern of CycA can be observed (G). In embryos that undergo the 16th cell cycle, HA-Rca1 starts to be degraded in a pattern reminiscent but not identical to that of CycA (compare [H] with [I] and [J] with [K]). HA-Rca1 and CycA were absent in most G1 cells of the epidermal cell layer at later stages (L and M). (N–P) HA-Rca1 is disappearing from cells during G1. A group of cells undergoing mitosis of the 16th cell cycle is seen in the right part of this region. Cells in different stages of mitosis are visible (O), but HA-Rca1 is present throughout this region (N). In a group of cells that had completed mitosis 16 and that are in G1 of cycle 17 (encircled by a dashed line in [N], [O], and [P]), CycA is completely degraded (P), but only a fraction of those cells had degraded Rca1 (N).

3F). However, no change in normal mitotic progression was observed, and the mitotic degradation pattern of CycA was normal (Figure 3G). HA-Rca1 was present throughout the cell cycle without signs of degradation. However, during the 16th cell cycle, HA-Rca1 degradation started in a pattern that was similar but not identical to that of CycA (Figures 3H–3K). HA-Rca1 and CycA were absent in most G1 cells of the epidermal cell layer at later stages (Figures 3L and 3M). We compared degradation of Rca1 and CycA in a region of the embryo that undergoes mitosis 16 (Figures 3N–3P). HA-Rca1 was present throughout the right part of this magnified region, where cells in different stages of mitosis are visible (Figures 3N and 3O). We saw degradation of CycA in a group of cells that had completed mitosis 16 and were in G1 of cycle 17 (Figures 3N–3P, dashed line), but only a fraction had degraded HA-Rca1 (Figure 3N). We conclude that Rca1 is not degraded during mitosis but disappears during G1.

The embryos in which HA-Rca1 was expressed throughout development using the *arm*-Gal4 driver line hatched, and larvae developed to the pupal stage, but flies failed to eclose (data not shown), typical for defects during imaginal disc development. Thus, while Rca1 overexpression does not influence cell cycle progression during embryogenesis, it does perturb development at later stages.

Rca1 Suppresses Cdh1^{Fzr}

The degradation of CycA in *rca1* mutants apparently occurs in late interphase before cells would enter mitosis. Normally, CycA is stable in cellularized embryos during interphase (Edgar et al., 1994). CycA degradation starts in metaphase and persists through the reminder of mitosis and G1 (Lehner and O'Farrell, 1989). The activities that cause CycA degradation are still poorly understood (Kaspar et al., 2001). Genetic data indicate that Cdc20^{Fzy} and Cdh1^{Fzr} are required for the degradation of CycA in mitosis and G1, respectively. While Cdc20^{Fzy} is expressed throughout embryogenesis, *Cdh1^{Fzr}* transcript is upregulated during stage 11, consistent with its function during G1. In *fzy* mutants, ectodermal cells arrest with high CycA levels in metaphase of cycle 16 (Dawson et al., 1995; Sigrist et al., 1995). *fzr* mutants are unable to keep mitotic cyclin levels low during G1, resulting in an additional S phase and mitosis (Sigrist and Lehner, 1997). Overexpression of Cdc20^{Fzy} does not perturb cell cycle progression (Sigrist and Lehner, 1997; and our own observations), while overexpression of Cdh1^{Fzr} causes premature cyclin degradation (Sigrist and Lehner, 1997). The overexpression of Cdh1^{Fzr} by the *prd*-Gal4 driver line results in premature CycB and CycA degradation (Sigrist and Lehner, 1997; and Figures 4A–4C). Cells fail to execute mitosis 16, and, consequently, fewer cells are visible in those segments (Figures 4D and 4E). This phenotype is strikingly similar to the *rca1* phenotype. Thus, a possible explanation of the *rca1* mutant phenotype would be an abnormal activity of Cdh1^{Fzr} in interphase of cell cycle 16.

We therefore tested whether Rca1 suppresses the effect of ectopic Cdh1^{Fzr}. We coexpressed *HA-rca1* and *fzr* transgenes using the *prd*-Gal4 driver line (Figures 4F–4J). In these embryos, CycA is no longer degraded prematurely in the *fzr*-expressing segments, and the overall pattern of CycA degradation looks identical to that of wt embryos (Figures 4G and 4H). In older embryos, similar cell numbers were found in all segments (Figures 4I and 4J), indicating that all cells were able

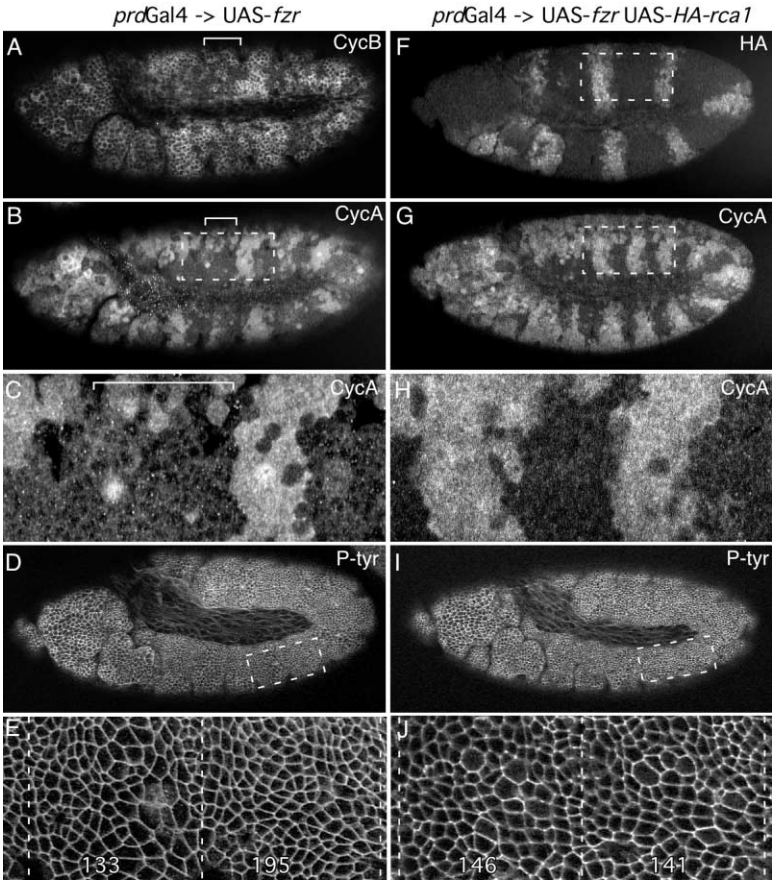

Figure 4. HA-Rca1 Can Suppress the Effects of Cdh1^{Fzr} Overexpression

(A–E) Embryos in which *fzr* was overexpressed using the *prd*-Gal4 driver line. Brackets indicate segments where Cdh1^{Fzr} is expressed. In stage 11 embryos, CycB (A) and CycA (B and C) are prematurely degraded in the cells that overexpress Cdh1^{Fzr}. This results in less mitotic divisions, which is visualized in stage 12 embryos by P-tyr staining (D and E).

(F–J) In embryos overexpressing Cdh1^{Fzr} and HA-Rca1 (visualized by HA staining in [F]), no premature CycA degradation is observed (G and H). At stage 12, similar cell numbers are present in neighboring segments.

to undergo a normal proliferation program. Thus, Rca1 overexpression counteracts the effect of Cdh1^{Fzr} overexpression.

fzr Is Epistatic to *rca1*

Cdh1^{Fzr} is a regulatory subunit of the APC. The inhibition of ectopic Cdh1^{Fzr} activity by Rca1 overexpression could be explained if Rca1 inhibits either Cdh1^{Fzr} function or the activity of the core APC. Accordingly, in the absence of *rca1* function, either Cdh1^{Fzr}-dependent APC activity or APC activity regulated by other means would cause the premature cyclin degradation.

To distinguish between these possibilities, we analyzed *fzr;rca1* double mutants. If Rca1 affects APC activity independently of Cdh1^{Fzr}, we would expect that the double mutants continue to degrade cyclins prematurely. In the case that Rca1 is specifically preventing Cdh1^{Fzr}-associated activity during interphase 16, the double mutant should enter mitosis 16, since APC-Cdh1^{Fzr} activity is absent.

We used the only available *fzr* mutation, a small deficiency [Df(1)bi-D3] removing *fzr* and also the gene *hindsight* (*hnt*), that is required for the retraction of the germband (Frank and Rushlow, 1996) but has no influence on cell cycle progression (Sigrist and Lehner, 1997). We compared the cell density of wt, *rca1*, and *fzr* mutants with those of *fzr;rca1* double-mutant embryos at the beginning of germband retraction, when all epidermal cells in wt embryos have completed mitosis 16 (Figures 5A–5L). At this stage, *fzr* mutants also had completed mitosis 16, and the cell density was comparable to that of wt (Figures 5G–5I). In contrast, *rca1* mutant embryos displayed reduced cell numbers, since they failed to enter mitosis 16 (Figures 5D–5F). Finally, in *fzr;rca1* double-mutant embryos, cell density was again similar to wt embryos, indicating that these embryos had gone through mitosis 16 (Figures 5J–5L).

Thus, in *fzr;rca1* double mutants, the *rca1* phenotype is suppressed, indicating that the premature degradation observed in *rca1* mutants is mediated by Cdh1^{Fzr}. This suggests that Rca1 functions by inhibiting APC-Cdh1^{Fzr} activity during interphase. In *rca1* mutants, this complex would be active prior to mitosis and cause premature mitotic cyclin degradation, resulting in a failure to enter mitosis. In the double mutant, APC-Cdh1^{Fzr} activity is not present, and Rca1 is not required to prevent premature cyclin destruction.

Rca1 and CycE Are Partially Redundant in Their Ability to Inhibit Cdh1^{Fzr}

The activity of Cdh1^{Fzr} is negatively controlled by Cdk-mediated phosphorylation. Both Cdk1 and Cdk2 kinase activities have been implicated in Cdh1 phosphorylation (Blanco et al., 2000; Jaspersen et al., 1999; Listovsky et al., 2000; Sorensen et al., 2001; Zachariae et al., 1998). During the first 15 divisions, CycE/Cdk2 kinase activity is present throughout the cell cycle. It declines during cell cycle 16, caused by the downregulation of *CycE*

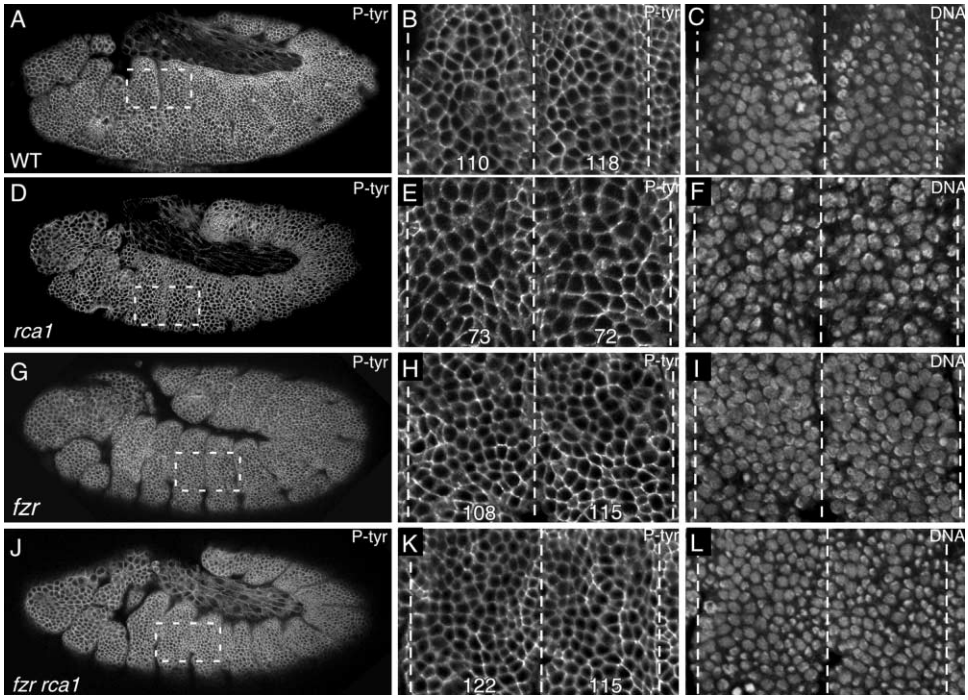

Figure 5. *fzr* Is Epistatic to *rca1*

(A–J) Comparison of cell density in wt (A–C), *rca1* (D–F), *fzr* (G–I), and *fzr;rca1* (J–L) stage 12 embryos. All embryos are stained with the P-tyr antibody and for DNA. At this stage, epidermal cells normally have gone through mitosis 16 and are in interphase of the 17th cell cycle. Thus, no mitotic figures are visible in the DNA channel (C). In *fzr* mutants, epidermal cells perform an extra cell cycle, but in the embryo shown, this extra division has not been initiated. Thus, cell numbers in this embryo are comparable to wt (G–I). In contrast, *rca1* mutant embryos show reduced cell numbers, since they fail to undergo mitosis 16 (D–F). In *fzr;rca1* double-mutant embryos cell density is similar to wt (J–L), demonstrating that mitosis 16 was executed. The suppression of the *rca1* mutant phenotype in *fzr;rca1* double mutants shows that *fzr* is epistatic to *rca1* and indicates that the premature destruction of mitotic cyclins observed in *rca1* mutants is mediated by Cdh1^{Fzr}.

transcription and the upregulation of the Cdk2-specific inhibitor *dacapo* (*dap)* (de Nooij et al., 1996; Lane et al., 1996). Thus, Cdh1^{Fzr} is not inhibited by CycE/Cdk2 activity during later stages of the 16th cell cycle. However, overexpressed CycE was able to suppress the effects of ectopic Cdh1^{Fzr} during cell cycle 16 (Sigrist and Lehner, 1997). To analyze whether CycE is also able to compensate for the lack of *rca1* function, we overexpressed CycE in *rca1* mutant embryos using the *prd*-Gal drive line (Figures 6A and 6B). In segments overexpressing CycE, higher cell densities were observed (Figures 6C and 6D). This demonstrates that CycE is able to suppress the *rca1* mutant phenotype, presumably by its negative influence on Cdh1^{Fzr} activity.

These data would suggest that CycE and Rca1 have overlapping functions in Cdh1^{Fzr} inhibition. Thus, the requirement for *rca1* only becomes visible when CycE levels decline during the 16th cell cycle. However, CycE and Rca1 are only partially redundant. This can be concluded from *rca1;CycA* double-mutant embryos. In *CycA* mutants, very low levels of CycA are already present during the 15th cell cycle but these levels are still sufficient to execute mitosis 15, and cells arrest before mitosis 16 (Lehner and O'Farrell, 1989). Thus, cell numbers are similar to *rca1* mutant embryos and reduced compared with those of wt (Figures 6E–6J). Interestingly, *rca1;CycA* double mutants have even fewer cells (Figures 6K and 6L). Apparently, these double-mutant embryos failed to execute mitosis 15, likely caused by a further reduction in CycA levels due to the absence of *rca1*. This shows that Rca1 is active at earlier cell cycles and becomes essential when CycA levels are reduced. Under these circumstances, CycE that is present during cycle 15 is apparently not sufficient to prevent excessive CycA degradation.

Rca1 and Cdh1^{Fzr} Interact Biochemically

Our genetic data indicate that Rca1 inhibits APC-Cdh1^{Fzr} function. To test whether Rca1 is in a physical complex with Cdh1^{Fzr}, we performed coimmunoprecipitation experiments. We used extracts from 6–8 hr old embryos in which HA-Rca1 was overexpressed using the *arm*-Gal4 driver line. A single band, corresponding to the HA-Rca1 protein, can be detected on Western blots of this extract using HA antibodies (Figure 7A, lane1). HA-Rca1 was efficiently immunoprecipitated by HA antibodies (lane 2). Control precipitations without HA antibodies (lane 3) or from wt extracts (lane 4) show that the immunoprecipitations were specific.

We then looked for coprecipitation of CycA, Cdc20^{Fzy}, Cdh1^{Fzr}, and Cdc27, a subunit of the APC. We were not able to detect CycA coprecipitating with HA-Rca1, indicating that they are not elements of a stable complex under these experimental conditions (data not shown). We also failed to see an interaction with Cdc20^{Fzy} (data not shown). In contrast, coprecipitations were seen with HA-Rca1 and Cdh1^{Fzr} and Cdc27 (Figure 7B). Both proteins were only present in the HA precipitations using

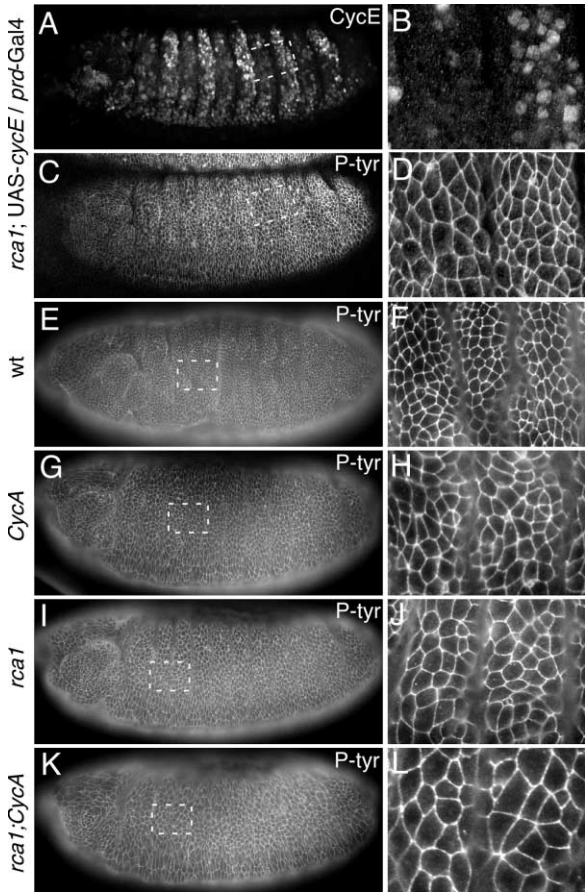

Figure 6. *CycE* and *rca1* Are Partially Redundant in Their Ability to Inhibit Cdh1^{Fzr}

(A–D) CycE can rescue the *rca1* mutant phenotype. (A and B) CycE was expressed using the *prd*-Gal4 drive line in every second segment. In a stage 13 *rca1* mutant embryo, endogenous and overexpressed CycE is visualized by CycE antibody staining. P-tyr staining of the same embryo (C and D) shows that segments overexpressing CycE have a higher cell density than nonexpressing segments.

(E and L) *rca1;CycA* double mutants arrest in the 15th cell cycle. At stage 13, cell densities were compared between wt (E and F), *CycA* (G and H), *rca1* (I and J), and *rca1;CycA* (K and L) embryos. All embryos are stained with the P-tyr antibody. Cell numbers in *CycA* (G and H) and *rca1* (I and J) mutant embryos are similar and reduced compared with wt (E and F), since both mutants fail to undergo mitosis 16. In *rca1;CycA* double mutants, cell numbers are further reduced, indicating that these embryos failed to execute mitosis 15 (K and L).

extracts from HA-Rca1-expressing embryos (Figure 7B, lane 2) but not from wt embryos (lane 4), although Cdh1^{Fzr} and Cdc27 were present in this wt extract (lane 5).

We also tested whether in vitro-translated Cdh1^{Fzr} and Rca1 can interact using reticulocyte lysates primed with *HA-Cdh1^{Fzr}* and *rca1* RNA (Figures 7C and 7D). We could see specific coimmunprecipitation of HA-Cdh1^{Fzr} and Rca1 in this system. This indicates that no other *Drosophila* protein is required for the complex formation between Rca1 and Cdh1^{Fzr}. However, we cannot rule out that other proteins present in the reticulocyte lysate are required for this interaction. In summary, these data demonstrate that Rca1 is in a physical complex with Cdh1^{Fzr} and an APC component, Cdc27.

Discussion

The results presented here show that Rca1 is an important regulator of mitotic entry. We show that Rca1 is required to inhibit Cdh1^{Fzr}-dependent APC activity during the G2 phase of the cell cycle. We propose that such a function is required for all cells in which kinase activity is low during G2.

In *rca1* mutants, levels of mitotic cyclins are reduced during interphase of the 16th cell cycle. This finding is in contrast to a previous study in which no differences in CycA levels were found (Dong et al., 1997). However, this premature cyclin disappearance became obvious only when we compared mutant and rescued segments in a given embryo and was more difficult to detect when mutant and wt embryos were compared. The lower levels of mitotic cyclins were not caused by changes in cyclin transcription or translation, since mitotic cyclins accumulate normally at the beginning of cell cycle 16. Mitotic cyclins are usually stable in interphase cells of cellularized *Drosophila* embryos (Edgar et al., 1994). We therefore conclude that their disappearance in *rca1* mutants is caused by premature degradation. The remaining cyclin levels are apparently not sufficient to allow entry into mitosis. In *Drosophila*, CycA and CycB are cytoplasmic during interphase and accumulate in the nucleus only during prophase (Huang and Raff, 1999; Lehner and O'Farrell, 1989). It has been speculated that the nuclear accumulation of mitotic cyclins is required for certain mitotic events like DNA condensation. Rca1 is a nuclear protein and could be required to prevent degradation of mitotic cyclins, specifically in the nucleus. Another possibility is that Rca1 sequesters parts of the degradation machinery in the nucleus away from the bulk of mitotic cyclins present in the cytoplasm.

In *rca1* mutant embryos, residual levels of cytoplasmic CycA and CycB are visible. Supplying additional CycA (but not CycB; data not shown) was sufficient to rescue the mitotic failure of *rca1* mutants. This demonstrates that CycA is the crucial mitotic factor missing in *rca1* mutant embryos.

The requirements for *rca1* function are not restricted to embryogenesis. Clonal analysis of *rca1* function shows that imaginal cells lacking *rca1* also have reduced cyclin levels and fail to proliferate normally. In these cells, large nuclei were found typical for cells undergoing endocycles. An overreplication has been reported in imaginal discs lacking Cdk1 activity (Hayashi, 1996). Since mitotic cyclins are degraded in *rca1* mutant cells, we expect low Cdk1 kinase activity that would result in the lack of proliferation and could result in overreplication of the genome. A detailed analysis of the DNA content and DNA replication pattern will reveal whether *rca1* mutant cells similarly undergo endocycles.

The APC targets cyclins for degradation, and its activity is normally restricted to mitotic stages and G1 (Amon et al., 1994; Brandeis and Hunt, 1996). However, in *rca1* mutants, we observe premature cyclin destruction during G2, indicating that Rca1 is required to inhibit the APC and thus ensures high mitotic cyclin levels for mitotic entry.

On a molecular level, Rca1 could inhibit APC ubiquitin ligase activity directly, or it might specifically prevent activation of the APC by Cdc20^{Fzy} and Cdh1^{Fzr}. Several

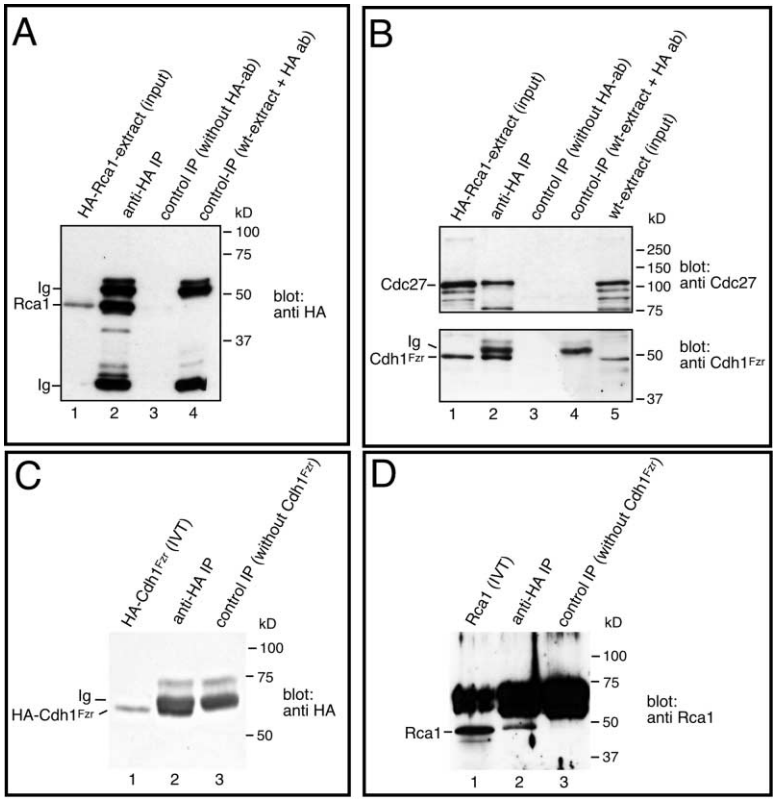

Figure 7. Rca1 Interacts Biochemically with Cdh1^{Fzr} and Cdc27

(A) Immunoprecipitation of HA-Rca1 from embryos expressing HA-Rca1 under the control of the *arm*-Gal4 driver line. 6-to 8-hr-old embryos were homogenized (lane 1) and HA-Rca1 was precipitated using HA antibodies (lane 2). Control precipitations either without antibody (lane 3) or with wt embryos (lane 4) do not show the HA-Rca1 band.

(B) Cdh1^{Fzr} and Cdc27 coprecipitate with HA-Rca1. HA-Rca1 was precipitated as in (A), and the blot was probed either with Cdc27 (upper blot) or Cdh1^{Fzr} (lower blot) antibodies. Cdh1^{Fzr} and Cdc27 are present in the embryonic extracts (lane 1) and coprecipitated with HA-Rca1 (lane 2). In control precipitations without HA antibody (lane 3) or with wt embryos (lane 4), both proteins are not detected but are present in wt embryos (lane 5).

(C) Immunoprecipitation of in vitro-translated HA-Cdh1^{Fzr}. Equal volumes of in vitro translated HA-Cdh1^{Fzr} and Rca1 were mixed and incubated for 20 min. HA-Cdh1^{Fzr} was immunoprecipitated using HA antibodies (lane 2). Control precipitations without antibody (lane 3) did not show the HA-Cdh1^{Fzr} band. Lane 1 shows in vitro translated HA-Cdh1^{Fzr} for comparison.

(D) Rca1 coprecipitates with HA-Cdh1^{Fzr}. In vitro translated HA-Cdh1^{Fzr} was precipitated as in (C), and the blot was probed with the Rca1 antibody. Rca1 coprecipitates with HA-Cdh1^{Fzr} (lane2) but is not present in control precipitations without HA antibody (lane 3). Lane 1 shows in vitro translated Rca1 for comparison.

lines of evidence suggest that Rca1 is a specific inhibitor of Cdh1^{Fzr}-dependent APC activity and does not affect APC-Cdc20^{Fzy}. First of all, we show that premature degradation of cyclins in *rca1* mutants depends on *fzr* gene function. Embryos mutant for *fzr* and *rca1* do not degrade cyclins prematurely, and mitosis 16 was restored. Thus, *fzr* is epistatic to *rca1*. In contrast, *rca1* is epistatic to *fzy* (P. O'Farrell, personal communication). In addition, overexpression studies also support the specificity of Rca1. Overexpression of Cdc20^{Fzy} is without consequences for cyclin levels and cell cycle progression (Sigrist and Lehner, 1997). In contrast, overexpression of Cdh1^{Fzr} resulted in premature cyclin degradation, like in *rca1* mutants (Figure 4; Sigrist and Lehner, 1997). The coexpression of Rca1 negated this phenotype, indicating that Cdh1^{Fzr} and Rca1 oppose each other.

During the first 16 cell cycles in *Drosophila*, Cdc20^{Fzy} is thought to be the major APC-activating protein, since Cdh1^{Fzr} is present at higher levels only at later stages. Uniform overexpression of HA-Rca1 had no influence on cell cycle progression or cyclin degradation during the first 16 divisions, indicating that Rca1 did not inhibit Cdc20^{Fzy}-dependent APC activity.

Ubiquitous overexpression of HA-Rca1 also had no influence on the establishment of the G1 state. This is in contrast to *fzr* mutants that fail to downregulate mitotic cyclins levels after mitosis 16 and that do not establish a G1 phase. However, HA-Rca1 itself is degraded in G1 cells and thus cannot influence Cdh1^{Fzr} function. We also suggest that Rca1 activity is subjected to cell cycle-specific regulation and expect that Rca1 function is downregulated during mitosis to allow Cdh1^{Fzr} activity during later stages of mitosis and at the beginning of G1.

A biochemical interaction between Rca1 and Cdh1^{Fzr} was seen in coimmunoprecipitation experiments. Using embryonic extracts, we showed that both proteins are present in a complex. We could not see any association with Cdc20^{Fzy} (data not shown). In addition, an interaction of Rca1 and Cdh1^{Fzr} was seen using reticulocyte-translated proteins, showing that no other *Drosophila* protein is required for complex formation. However, we cannot rule out that other proteins are required to mediate the interaction between Rca1 and Cdh1^{Fzr}. The complex precipitated from embryonic extracts also contained the APC component Cdc27. Thus, Rca1 might be able to inhibit preformed APC-Cdh1^{Fzr} complexes. Additionally it could prevent a fruitful association of Cdh1^{Fzr} with the APC. Regardless of the exact biochemical composition of the Rca1-containing complex, all of our data support the conclusion that Rca1 is a specific inhibitor of Cdh1^{Fzr}-dependent APC activity. This function of Rca1 is necessary during the G2 stage of the cell cycle to prevent a premature activation of the APC-Cdh1^{Fzr} complex.

Cdh1 is also regulated by phosphorylation (Kotani et al., 1999; Kramer et al., 2000). Only unphosphorylated Cdh1 can bind to and activate the APC, and several kinases have been implicated in the phosphorylation of Cdh1, including Cdk1 and Cdk2 (Blanco et al., 2000; Jaspersen et al., 1999; Listovsky et al., 2000; Sorensen et al., 2001; Zachariae et al., 1998). Accordingly, no Cdh1-dependent APC activity was found during S phase and

early mitotic stages. When degradation of mitotic cyclins abate Cdk1 activity after the metaphase-anaphase transition, unphosphorylated Cdh1 is thought to activate the APC. This activity is then maintained during the G1 state and turned off when cells start to accumulate Cdk2 activity at the G1-S transition (Lukas et al., 1999). The G2 state is also characterized by low Cdk kinase activity, yet not in all cell cycles. In *Drosophila*, Cdk1 is inhibited during G2 stages by tyrosine phosphorylation (Edgar et al., 1994). But during the first 15 divisions, Cdk kinase activity is provided by CycE/Cdk2 activity that is present throughout the cell cycle, including G2 (Knoblich et al., 1994). During cell cycle 16, CycE/Cdk2 kinase activity drops, since *CycE* mRNA is downregulated and the CycE/Cdk2 inhibitor Dacapo is upregulated (de Nooij et al., 1996; Knoblich et al., 1994; Lane et al., 1996). Thus, in G2 of cell cycle 16, Cdk1 as well as Cdk2 activity is expected to be low. At this stage, phosphorylation of $Cdh1^{Fzr}$ cannot prevent its association with the APC, and the requirements for Rca1 become evident. Accordingly, we could show that Rca1 was dispensable when CycE was overexpressed during cell cycle 16. Thus, phosphorylation and interaction with Rca1 can control $Cdh1^{Fzr}$ activity during G2. However, Rca1- and CycE-dependent phosphorylation of the APC are not completely redundant. In *CycA* mutant embryos, maternally provided CycA is normally sufficient to allow execution of mitosis 15 despite low CycA levels. In *rca1;CycA* double mutants, cells arrest before mitosis 15, likely caused by a further reduction in CycA. During this stage, CycE is still present but apparently cannot substitute completely for the lack of *rca1* function.

We expect that Cdh1 inhibition by an Rca1-like function is also required in other organisms. In human cells, Cdh1 phosphorylation is also low when most cells are in late S phase and G2, but only low APC activity was detected (Kramer et al., 2000). Thus, human cells must also have a mechanism preventing APC-Cdh1 activation when Cdh1 phosphorylation is low.

Recently, potential homologs of Rca1 have been identified in *Xenopus*, mouse, and humans and named Emi1 (Reimann et al., 2001). In this study, the authors provide evidence that Emi1 from *Xenopus* inhibits Cdc20 activity in the *Xenopus* system. In contrast, our data show that Rca1 specifically inhibits APC-$Cdh1^{Fzr}$, but not APC-$Cdc20^{Fzy}$ activity. Databank searches revealed that the *Drosophila* genome does not contain an additional protein that resembles Emi1 or Rca1. We thus believe that Rca1 has specificity different than that of Emi1. This difference might reflect the manner in which cell cycle progression is controlled in these organisms. In *Drosophila*, CycA- and CycB-containing Cdk1 complexes are targets for inhibitory phosphorylation (Campbell et al., 1995; Edgar et al., 1994). The CycA/Cdk1 complex in *Xenopus*, on the other hand, is not subject to this modification, and one can expect Cdk1 activity even in G2 (Clarke et al., 1992; Devault et al., 1992). That could result in the phosphorylation of the APC and activation by Cdc20, even before the spindle checkpoint is activated. Thus, Emi1 might have been adapted to these specific requirements in *Xenopus* to suppress Cdc20-dependent APC function before mitosis. Rca1, on the other hand, is required to prevent APC-$Cdh1^{Fzr}$ activity when Cdk1 kinase activity is low in G2, an environment that is permissive for APC-Cdh1 complex formation. Significantly, recent work on the human form of Emi1 revealed that this protein inhibits the APC-Cdh1 complex (P. Jackson, personal communication). In *S. pombe*, which is also characterized by low Cdk kinase activity in G2, premature APC-Ste9 (the Cdh1 homolog) activity is probably prevented by very low Ste9 protein levels during G2 (Blanco et al., 2000). Thus, different ways of preventing premature APC activation at the G2 stage have been selected in various organisms and by different cell types within an organism. Our data reveal the importance of APC downregulation by the Rca1 protein that is specific for the APC-$Cdh1^{Fzr}$ activity. In addition to the Emi1/Rca1 class of proteins, inhibition of the APC-Cdh1 complex is also mediated by a protein related to Mad2 protein (Chen and Fang, 2001; Pfleger et al., 2001). The biological role of this inhibition has not been elucidated so far.

Thus, a number of different mechanisms regulating the activity of the APC-Cdh1 have been identified recently. It has been shown that APC activity is restricted to mitotic stages and G1. Our data on Rca1 demonstrate a novel control of APC-Cdh1 activity that is necessary to prevent unwanted APC activity at the G2 stage. We expect that this mechanism of controlling APC-Cdh1 activity is also involved in the downregulation of APC-Cdh1 activity at the G1-S transition.

Experimental Procedures

Fly Stocks, Transgenics, and Genetics

The *rca1* mutant flies and *rca1* cDNA were kindly provided by Larry Zipursky. The analysis of the *rca1* mutant phenotype was done with the heteroallelic combination $rca1^2/rca1^3$. Both strains were balanced over *CyO-wg-lacZ* to identify homozygous embryos by the absence of β-galactosidase staining. All other experiments were done with the $rca1^2$ allele. The UAS-*HA-rca1* transgenic flies were constructed using standard molecular and fly transformation techniques. The *paired*-Gal4 strain was described before (Xiao et al., 1996). The UAS-*HA-cycA* strain was generated by Axel Dienemann (our own unpublished data). UAS-*HA-rca1* overexpression was achieved with either the *paired*-Gal4 or *armadillo*-Gal4 (Sanson et al., 1996) driver line. Mitotic *rca1* clones were induced using the FLP/FRT technique (Xu and Rubin, 1993). The $rca1^2$ allele was recombined onto the *FRT40A* chromosome. The resulting $FRT40Arca1^2$/*CyO-wg-lacZ* flies were crossed to flies carrying *hs-FLP1.22*; *FRT40A2xGFP* (a gift from Thomas Klein). Clones were induced 48 hr after egg deposition (AED) by a 30 min heat shock at 37°C. Discs were analyzed 72 hr after clone induction. For the control clones, the *FRT40A2xGFP* without the *rca1* mutation was used.

The UAS-*fzr* and UAS-*fzy* strains were described before (Sigrist and Lehner, 1997). For coexpression of *HA-rca1* and *fzr*, UAS-*HA-rca1*; UAS-*fzr*/*CyO-wg-lacZ* flies were generated. The deficiency *Df(1)bi-D3/FM7* (w^+ *ftz-lacZ*), which also removes the gene *hindsight* (*hnt*), was used to analyze the *fzr* mutant phenotype and to generate *fzr;rca1* double mutants.

Antibodies, Immunofluorescence and Microscopy

The Rca1 and Fzr antibodies were generated in rats immunized with bacterially produced GST-fusion proteins. Antibodies against CycA and CycB have been described previously (Kaspar et al., 2001). The Cdc27 antibody is a gift from Jordan Raff, who also kindly provided us with Fzr antibodies (J. Raff, personal communication). The Fzy antibody is a gift from Ian Dawson, and the CycE antibody is a gift from Helena Richardson. The antibodies against HA, β-galactosidase, PH3, and Phosphotyrosin are from Roche, Cappel, and Upstate Biotechnology, respectively. Secondary antibodies conjugated with Alexa488, Rhodamin, Cy5, and HRP were obtained from Molecular Probes and Jackson Research Laboratories. DNA was stained with

Hoechst or propidiumiodid. Immunofluorescent pictures were taken either on a Zeiss Axiovert 10 using a CCD Camera (Quantix, Photometrics) or a confocal microscope (Leica).

In Vitro Translations, Immunoprecipitations, and Immunoblotting

The *rca1* ORF was cloned in a SP64 based vector with and without the HA tag and the *Xenopus* β-globin 5′-leader sequence. The same was done with *fzr*. Transcription in vitro and translation in reticulocyte lysate (Promega) were performed as described (Foley et al., 1999). For immunoprecipitations with embryonic extracts 6- to 8-hr-old embryos were homogenized in immunoprecipitation buffer (IP buffer: 10 mM Tris, pH 7.5, 80 mM K-β-glycerophosphate, pH 7.3, 20 mM EGTA, pH 8.0, 15 mM $MgCl_2$, 0.5 mM DTT, 2 mM Na_3VO_4, 10% glycerol, 0.1% NP40) and incubated with 10 μl protein G sepharose beads (Pharmacia) and 4 μl HA-antibody (5 mg/ml) over night at 4°C. The immunoprecipitation was then washed three times with IP buffer, the beads boiled in loading buffer, and proteins separated by SDS-PAGE. The gel was blotted on nitrocellulose, Western blots performed and developed using the ECL-system (Amersham). For immunoprecipitations with in vitro-translated proteins, equal volumes of translation reactions were mixed and incubated for 20 min at room temperature and processed as above.

For Western blots without preceding immunoprecipitation, staged embryos were methanol fixed. A given number of embryos was then homogenized in loading buffer and boiled for 5 min. Usually, ten embryos per lane were loaded.

Acknowledgments

We thank Axel Dienemann and Edan Foley for comments on the manuscript, Larry Zipursky for the *rca1* fly stocks and *rca1* cDNA, Jordan Raff, Ian Dawson, and Helena Richardson for antibodies, Christian Lehner for UAS-*fzr* and UAS-*fzy* flies and *fzr* cDNA, and Jordan Raff as well as Pat O'Farrell for sharing unpublished results.

Received July 5, 2001; revised November 12, 2001.

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